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Mutants of *Erwinia chrysanthemi* Defective in Secretion of Pectinase and Cellulase

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***Erwinia chrysanthemi* produced several pectate lyases (EC 4.2.2.2) and endocellulases (EC 3.2.1.4) which were largely secreted into the culture medium. Mutants deficient in the secretion mechanism for these enzymes were obtained by chemical and insertion mutagenesis. Further study of one such mutant revealed that both enzyme activities were retained simultaneously within the periplasmic space.**

Erwinia chrysanthemi is a causal agent of soft rot in many plants (20) and has served as a model in studies aimed at defining the determinants of plant pathogenicity and the mechanisms involved in plant cell wall degradation. The bacterium produces several enzymes that depolymerize various plant cell wall constituents, including pectinases, cellulases, and proteases (4; C. Wandersman et al., manuscript in preparation).

At least 14 pectate lyase (PL) isoenzymes (EC 4.2.2.2) with isoelectric points ranging from 4.4 to over 9.5 have been identified in culture supernatants of *E. chrysanthemi* by electrofocusing in polyacrylamide gels (1). Two endocellulases (Cx) (EC 3.2.1.4), active on carboxymethylcellulose, were also detected by electrophoresis of culture supernatants; one of these has been purified and characterized (M. H. Boyer et al., J. Biotechnol., in press).

Over the last decade, significant progress has been achieved in our understanding of the genetics of this bacterium. Genetic maps have been obtained by different means (3, 11, 18), and some strains have been shown to be susceptible to infection by phage Mu (8).

Although other workers have stated previously that PL is secreted by *E. chrysanthemi* (2, 5), the secretion of cellulases has never been demonstrated. Therefore, this current study was undertaken in order to explain how PL and Cx are transported out of the bacterial cell.

Bacterial strains used in this study (Table 1) were grown on GPGY medium, which is M9 medium (14) supplemented with 0.5% (wt/vol) glycerol, 0.5% polygalacturonic acid (grade III; Sigma Chemical Co.), 0.1% sodium galacturonate (Sigma), 0.1% yeast extract (Difco), and 2 mM isopropylthiogalactoside. PL, Cx, and β-galactosidase synthesis were induced in this medium.

To determine the levels of PL and Cx both inside the bacteria and present in the medium, liquid cultures of *E. chrysanthemi* were treated as follows. Cells were harvested by centrifugation at 6,000 × *g* for 15 min, and the supernatant was filtered with a Millipore filter (0.45-μm pore size). Bacteria were then lysed by suspension in 10 mM Tris hydrochloride containing lysozyme (80 μg/ml; Sigma) and 10 mM EDTA (whole-cell lysate). β-Galactosidase activity was assayed spectrophotometrically as described by Dobrogosz (6). PL was assayed by the thiobarbituric acid (TBA) method (19); one unit of activity corresponded to an increase of 0.1 absorbance unit under standard conditions (1). When assaying PL in periplasmic or cytoplasmic fractions, it was first necessary to saturate the EDTA with $CaCl_2$ to ensure an excess of the Ca^{2+} required for enzymatic activity. β-Lactamase (12) and Cx (23) were assayed by the cup-plate technique, which is based on radial diffusion of the enzymes into a substrate-bearing agar gel slab. This technique is reported to be both sensitive and quantitative (12, 23). All enzymes were assayed at 37°C.

We first examined the production and location of PL and Cx during the growth of *E. chrysanthemi* in liquid culture. Results obtained with strain TA1 (Fig. 1a and b) showed that PL and Cx were synthesized from the early exponential phase and that the rate of synthesis was increasing when the absorbance at 600 nm (A_{600}) was ca. 1.5, so that the total activity in the culture was maximum in the late-log phase (at A_{600} = 2). The differential rate of synthesis (first-order derivative of Fig. 1 curves) reached its maximum just before A_{600} = 2 for both activities. Total activity stabilized before the culture entered the stationary phase, which may be explained in terms of either cessation of synthesis or establishment of an equilibrium between synthesis and degradation-inactivation. These kinetics demonstrated that, during the second half of the exponential phase (when $A_{600} > 1.5$), PL and Cx were mainly extracellular. A transient intracellular pool was evident at one stage (Fig. 1a and b) which later disappeared before the onset of the stationary phase. At A_{600} = 3, more than 90% of the total activity of the culture was found in the supernatant (Fig. 1).

E. chrysanthemi PL and Cx activities thus appeared to be extracellular. However, the presence of the enzymes in the culture medium may be explained in terms of (i) nonspecific mechanisms, i.e., after cell lysis during growth, leakage through the outer membrane, or, alternatively, by (ii) a specific secretory system. If a nonspecific process was involved, intracellular proteins would have been found in the culture medium in the same ratio as PL and Cx. To resolve the question, β-galactosidase and β-lactamase were chosen as cytoplasmic and periplasmic enzyme markers, respectively, and were assayed in *E. chrysanthemi* cultures in parallel with PL and Cx. *E. chrysanthemi* was made β-lactamase positive by the transfer of plasmid pULB113, which derives

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TABLE 1. Bacterial strains used

Strains	Genotype and phenotype[a]	Source or reference
E. coli		
MXR(pULB113)	K-12 *galE recA thi* Δ(*pro-lac*) RP4::Mu3A Kan^r Amp^r Tet^r	21
MXR(Mu *cts*62)/F::mini-D108::Tn*9*	Ts *galE recA thi* Δ(*pro-lac*) Cam^r	A. Toussaint
MXR(Mu7701)	Ts *galE recA thi* Δ(*pro-lac*) Mu(G+) Δ(*kil-arm*) Kan^r	A. Toussaint
E. chrysanthemi		
3665	Wild type, isolated from dieffenbachia	M. Lemattre
3665-5	Out$^-$	NTG[b] mutagenesis of 3665
B374	Wild type	18
A24	Kan^r Out$^-$	Mutagenesis of B374 with Mu7701
Hfrq	Hfr Lac$^+$ Tet^r Tra$^+$ Nal^r	Hfr derived from strain 3937j (11)
TA1	Hfr Lac$^+$ Tet^r Tra$^+$ Nal^r Amp^r Kan^r	Conjugational transfer of pULB113 to Hfrq
34	Hfr Lac$^+$ Tet^r Tra$^+$ Nal^r Cam^r Out$^-$	Mutagenesis of Hfrq with mini-D108::Tn*9*
TA5	Hfr Lac$^+$ Tet^r Tra$^+$ Nal^r Cam^r Out$^-$ Amp^r Kan^r	Conjugational transfer of pULB113 to strain 34

[a] Abbreviations: Amp^r, ampicillin resistant (20 μg/ml); Cam^r, chloramphenicol resistant (20 μg/ml); Kan^r, kanamycin resistant (20 μg/ml); Nal^r, nalidixic acid resistant (25 μg/ml); Tet^r, tetracycline resistant (15 μg/ml); Out$^-$, secretion deficient (see text).

[b] NTG, *N*-Methyl-*N'*-nitro-*N*-nitrosoquanidine.

from RP4 and carries transposon Tn*1* encoding a TEM β-lactamase (10; Table 1, strain TA1). β-Lactamase and β-galactosidase were found to be largely cell associated throughout the cultivation period. Until A_{600} reached 2.5, activity in the supernatant remained below 10% of the total activity present in the culture. Similar results were obtained with two other wild-type strains, B374 and 3665. These data (not presented here) appear to exclude lysis of either whole

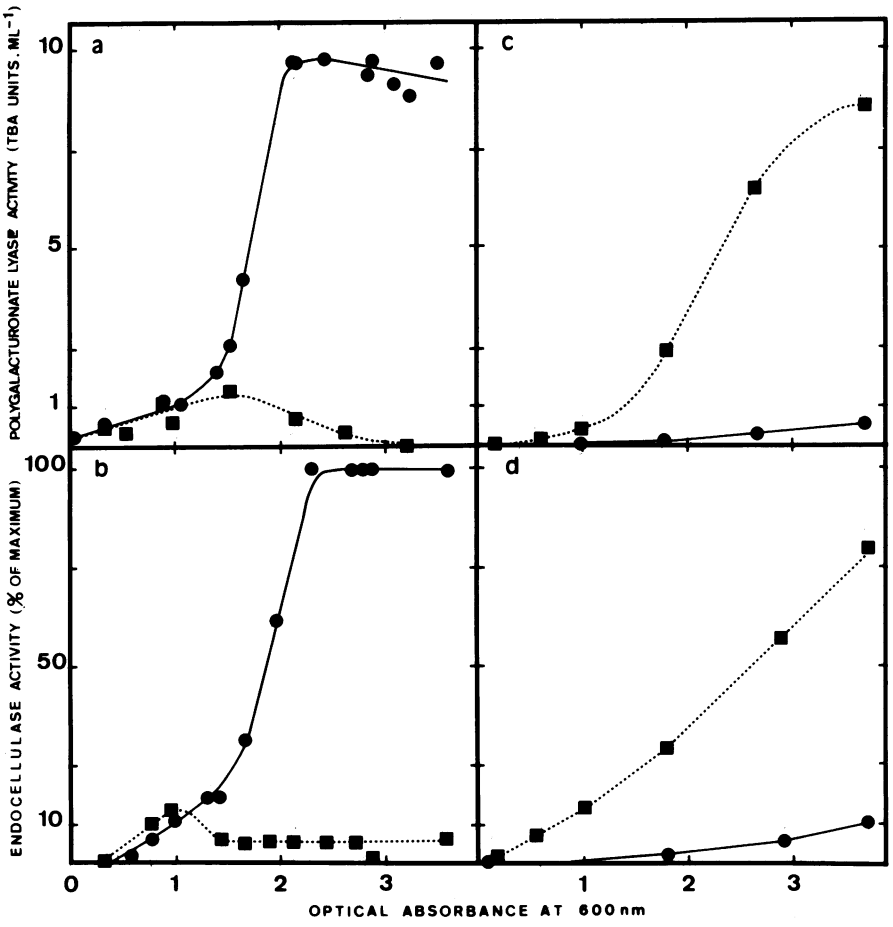

FIG. 1. Distribution of PL (or polygalacturonate lyase) (a and c) and Cx (b and d) during growth. Cultures were grown in GPGY medium, and cells were lysed as described in the text. Activities in the supernatant (●) and in the whole-cell lysate (■) were measured for the Out$^+$ strain TA1 (a and b) and the Out$^-$ strain TA5 (c and d).

cells or periplasm before the onset of the stationary phase and thereby suggest that PL and Cx were truly secreted during the exponential phase by a specific mechanism. This conclusion is supported by the isolation of mutants affected in PL and Cx secretion (Out^-).

Chemical mutagenesis of strains 3665 and Hfrq (Table 1) was performed with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (14) and ethyl methanesulfonate (11). Insertion mutagenesis was also undertaken with B374 and Hfrq, using several derivatives of phage Mu. *E. chrysanthemi* is sensitive to Mu having its G loop in the minus orientation [Mu(G−)] (8). Phage stocks were prepared by thermal induction (1a) of *Escherichia coli* strains dilysogenic for different Mu, mini-Mu, or mini-D108 phages carrying resistance genes for ampicillin, kanamycin, or chloramphenicol, together with a thermoinducible helper phage providing functions for the lytic cycle (17). The different stocks were used to infect *E. chrysanthemi* cells, and antibiotic resistance was used as the basis for selecting Mu, mini-Mu, or mini-D108 insertions in the chromosome. Over 9,000 such mutants were screened for acquisition of auxotrophy and for PL or Cx production. The proportion of auxotrophs was 1.1%, and actual auxotrophic requirements were variable. All mutants were replicated onto two types of differential media designed to reveal PL or Cx secretion around the colonies (Fig. 2). Mutant colonies lacking extracellular enzyme activity were readily identified. Among those resulting from the insertion of mini-Mu or mini-D108, only those mutants which did not lyse at 37°C were retained, i.e., probably not lysogenic for the Mu helper phage.

Preliminary experiments were undertaken to establish the presence and location of PL and Cx, using 27 of these mutants derived from strains Hfrq, 3665, and B374. Although the mutants had been selected as secretion deficient for either Cx or PL, all were still producing both enzymes, but both enzymes were bound to the cells (data not shown). Observation of this phenotype (Out^-) suggested that the isolated mutation(s) was in some way affecting the secretion rather than the synthesis of the enzymes. Moreover, this secretion system appeared to be common for both enzymes.

Several Out^- mutants, all derived from strains Hfrq, B374, or 3665, gave essentially similar results, and the present analysis describes one such Out^- mutant, strain TA5, which was otherwise isogenic to strain TA1 (Table 1).

A comparison between TA5 and TA1 was made first with respect to growth and the kinetics of PL, Cx, β-galactosidase, and β-lactamase synthesis in liquid culture. Both strains exhibited similar growth rates and achieved the same cell concentration at the stationary phase. Moreover, there was no difference between the strains with respect to synthesis and location of β-galactosidase and β-lactamase (data not shown). On the other hand, the distribution pattern of PL and Cx activities between the cells and the extracellular medium was reversed (Fig. 1c and d). Thus, throughout the entire exponential phase, the enzymes were not secreted and instead accumulated inside the bacterial cell. Although the differential rate of synthesis value for PL and Cx was lower in strain TA5, total activity at the end of the cultivation period was ca. 80% of that found in strain TA1 (Fig. 1). This confirms that the *out* mutation did not markedly impair either enzyme synthesis or viability and growth rate of the mutant.

To determine which step of the secretion process was suppressed in the Out^- mutant, we investigated the location of PL and Cx within the cells, using both strain TA5 and strain TA1. Cells were fractionated by three separate methods. The first involved the production of spheroplasts by a modification of the lysozyme-EDTA method (15). Bacteria were grown in GPGY medium to $A_{600} = 2$ and harvested by centrifugation, and the supernatant fraction was retained. The bacterial pellet was suspended in 10 mM Tris hydrochloride (pH 7.5) containing 1.5 M sucrose to a concentration of 7×10^9 cells per ml. Lysozyme (1.5 mg/ml) was added at 4°C with careful stirring, followed by 1.5 mM EDTA in 10 mM Tris hydrochloride (pH 7.5), added by using a peristaltic pump at a rate of 1 ml/min. Final concentrations were 0.5 M sucrose, 75 μg of lysozyme per ml, and 1 mM EDTA. The spheroplasts were protected with 10 mM $MgCl_2$, and the suspension was centrifuged (10,000 × *g*, 15 min, 4°C), the resultant supernatant corresponding to the periplasmic fraction. The pellet of spheroplasts was washed with 10 mM Tris hydrochloride (pH 7.5) containing 0.5 M sucrose and suspended in cold water to give the cytoplasmic-plus-membrane fraction. β-Galactosidase, β-lactamase, PL, and Cx were assayed in the different fractions (Table 2). Despite several attempts to improve spheroplast formation, it was not possible to achieve a full separation between cytoplasm and periplasm, possibly due to the fragility of *E. chrysanthemi* spheroplasts.

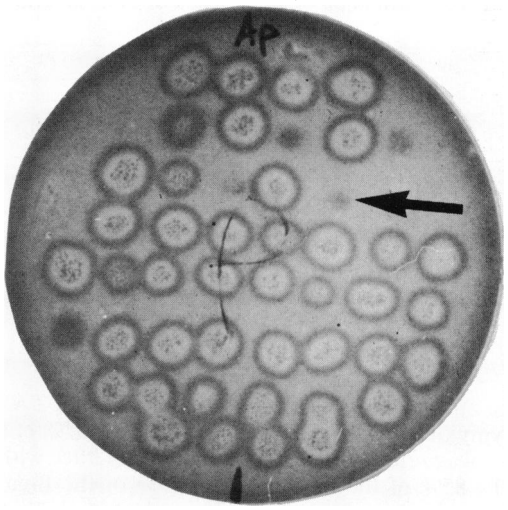

FIG. 2. Screening for Out^- mutants. Mutants were replicated on M9 medium (14) with 0.5% (wt/vol) sodium polygalacturonate, 0.5% (wt/vol) glycerol, 0.1% (wt/vol) yeast extract, 1 mM $MgSO_4$, 1 mM $CaCl_2$, and 1.5% agar. After 10 h of incubation at 30°C, the plates were flooded with a saturated solution of copper acetate, and patches of *E. chrysanthemi* secreting PL into the medium were surrounded by halos. Patches of the Out^- mutants lacked this halo (arrow). Mutants were replicated onto the same medium, but containing 0.5% (wt/vol) carboxymethylcellulose instead of polygalacturonate, to screen for extracellular production. After 24 h of incubation at 30°C, halos of Cx activity were revealed by flooding plates with 0.1% (wt/vol) Congo red for 15 min, followed by bleaching with 1 M NaCl (23).

Nevertheless, the data in Table 2 indicate that PL and Cx remained inside the cells of the Out^- mutant and were mostly confined to the periplasm, since the distribution of these enzyme activities was essentially the same as that of β-lactamase. Furthermore, the small proportion of PL and Cx bound to the cells of the Out^+ strain TA1 was also found in the periplasm.

These results were supported when a second fractionation method was adopted in which polymyxin B was used to

TABLE 2. Distribution of enzyme activities in subcellular fractions[a]

Enzyme	% Total activity in:					
	Cytoplasm		Periplasm		Supernatant	
	TA1	TA5	TA1	TA5	TA1	TA5
β-Galactosidase	40	42	54	52	6	6
β-Lactamase	22	18	76	75	2	7
PL	0	18	9	82	91	0
Cx	0	20	20	80	80	0

[a] Enzyme activities were assayed as described in the text and are expressed as percentage of total activities. Cells of the mutant strain TA5 and its wild-type progenitor TA1 were fractionated by a lysozyme-EDTA method as described in the text.

attack the outer membrane (7). After incubation of TA5 cells with polymyxin B (2 mg/ml) for 2 min at 37°C, 87% of the β-lactamase was released into the medium, together with 87% of the PL, 85% of the Cx, and only 15% of the β-galactosidase. Remaining enzyme activities were found inside the cells. Similar proportions were obtained with TA1, although the absolute levels of PL and Cx activities were much lower. Thus, the separation between cytoplasm and periplasm was more complete with this method.

The third fractionation method was used to investigate a possible bond between the enzymes, when located intracellularly, and the membrane. TA5 and TA1 cells were harvested by centrifugation at the end of the log phase, suspended in cold water to 1/50 of the initial volume, and sonicated (five 30-s pulses; instrument set at "low 3"; MSE apparatus). Cell disruption was followed microscopically, and membranes were pelleted by centrifugation with a swinging bucket rotor (100,000 × *g* maximum, 1 h, 4°C). With both strains, 87% of the β-galactosidase and 90% of the intracellular PL activity was found in the supernatant, the remainder being located in the membrane fraction. Approximately the same proportions were obtained for Cx and β-lactamase.

Thus, the fractionation experiments seem to indicate that, when intracellular, PL and Cx are located largely within the periplasmic space and are not membrane bound. The enzymes also seemed to accumulate within the periplasm of the Out$^-$ mutant.

Until recently, the secretion of protein into the extracellular growth medium by gram-negative bacteria has been considered as a rare, relatively undefined phenomenon. Protein secretion has now been reported in several gram-negative organisms including *E. coli* (hemolysin) (22), *Aeromonas hydrophila* (hemolysin, protease, and phospholipase) (9), and *Pseudomonas aeruginosa* (hemolysin and alkaline phosphatase [16] and exotoxin A [13]). Most of these proteins are involved in human pathogenic processes, and similarly, pectinases and cellulases seem to be determinants of *E. chrysanthemi* pathogenicity on plants (4). Indeed, Out$^-$ mutants, isolated from a wild-type virulent strain, were found to be avirulent for the test plant *Saintpaulia ionantha* (Andro, unpublished data). The *out* mutation(s) did not affect the synthesis of PL and Cx but resulted in their accumulation within the cells, apparently in the periplasmic space, where they were active. Furthermore, the molecular weight and the isolelectric point of the intracellular and extracellular forms of both enzymes appeared to be the same, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and by electrofocusing in polyacrylamide gel (data not shown) with either the Out$^-$ or the Out$^+$ strain. We conclude from this evidence, and also from the fact that several *out* mutations were obtained by insertion of transposable elements (Mu derivatives), that the mutations were not located in the structural gene of one of these enzymes but rather in gene(s) monitoring one or more steps of the secretion process. All of these steps should be shared by PL and Cx. But since the extracellular state of an *E. chrysanthemi* protease was not affected by the *out* mutation(s), other steps or another mechanism may exist. Do PL and Cx pass through the periplasm when they are secreted into the extracellular medium in the normal way, as it seems to be the case for hemolysin in *E. coli* (22)? Some indication of this is suggested by the transient presence of a periplasmic pool of these enzymes in Out$^+$ strains and by their accumulation within the periplasm of Out$^-$ mutants. Alternatively, the *out* mutation might have resulted in a major rerouting of the enzymes into the periplasm, whereas only a small proportion are normally rerouted in the Out$^+$ wild-type strains. Secretion of exotoxin A by *P. aeruginosa* may not involve passage of the protein through the periplasm but rather through junctions between the outer and inner membrane (13). Genetic mapping and molecular cloning of the *out* gene(s) are under way and should help to identify the proteins it encodes.

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